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Research Article

# Host Genetic Variants in the *Interleukin-6* Promoter Predict Poor Outcome in Patients with Estrogen Receptor-Positive, Node-Positive Breast Cancer

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## Abstract

**Interleukin-6 modulates immune response, estrogen production, and growth pathways in breast cancer. We evaluated the effect of several common, functional *interleukin-6* promoter variants in node-positive breast cancer patients enrolled on a multicenter, cooperative group, adjuvant chemotherapy trial to determine whether these variants were associated with clinical outcome overall and by estrogen receptor tumor phenotype. Genomic DNA and clinical data were collected from a clinical trial of adjuvant anthracycline-based chemotherapy followed by randomization to high-dose cyclophosphamide/thiotepa or observation (Intergroup Trial 0121). Genotyping for -174G>C (rs1800795), -597G>A (rs1800797), and -572G>C (rs1800796) was done by site-specific PCR and PyroSequencing, whereas the -373$A_nT_n$ repeat was directly sequenced. Log-rank tests and Cox modeling were used to compare outcomes by genotype/haplotype and other factors. Three hundred forty-six patients (64% of trial) had corresponding genotype/clinical data available and did not differ from overall trial participants. After adjustment, patients with estrogen receptor-positive tumors and genotypes 597 GG or 174 GG had significantly worse disease-free survival [hazard ratio (HR), 1.6; *P* = 0.02 and HR, 1.71; *P* = 0.007, respectively], whereas the 373 8A12T repeat appeared to be protective (HR, 0.62; *P* = 0.02). The presence of at least one copy of the haplotype ([-597G, -572G, -373[10A/11T], -174G]) was associated with worse disease-free survival (HR, 1.46; *P* = 0.04). Kaplan-Meier plots show that all patients in this group relapsed by 24 months from diagnosis. This poor-risk haplotype was quite common overall (estimated frequency, 0.20) and twice as frequent among Blacks (estimated frequency, 0.41).** [Cancer Res 2009;69(10):4184–91]

## Introduction

Despite recent advances in early detection methods and treatment, breast cancer remains a common and significant health problem in the United States (1). Women diagnosed with tumor involving both the breast and ≥10 involved axillary lymph nodes have a high risk for distant recurrence and half of all women will succumb to metastatic disease (2). Estrogen receptor (ER) positivity is typically considered a favorable prognostic marker (3). However, a substantial proportion of patients with ER+ tumors relapse despite endocrine therapy (4).

Tumor-based genetic profiling techniques are currently under development to identify poor-prognosis, ER+ subsets of patients. However, it is increasingly recognized that host markers, reflecting a variety of host-related processes including drug metabolism and tumor microenvironment, may also play an important role in modulating cancer behavior and response to therapy. Interleukin-6 (IL-6) is an inflammatory cytokine that has been implicated in the immune response to cancer (5) but also plays an important role in tumor progression and estrogen modulation (6). IL-6 provides direct signals for tumor cells, through specific cell membrane receptors triggering important intracellular signal pathways (7), leading to increased cell migration (8) and loss of apoptosis (9, 10). Additionally, IL-6 acts as a regulator of estrogen synthesis and aromatase activity in the peripheral tissues of normal and malignant breast tissue (6, 11). Functional polymorphisms in the promoter region of IL-6 control transcription and expression (12), providing genetic surrogates for host cytokine production that likely result in interindividual variation in tumor exposure to this cytokine.

We previously reported an association between the IL-6-174 GG genotype and decreased disease-free survival (DFS) and overall survival (OS) among women with ER+, node-positive breast cancer (13). We subsequently sought to further evaluate the full complement of functional variants in the IL-6 promoter [-572G>C (rs1800796), -597G>A (rs1800797), -174G>C (rs1800795), and the AT repeat at position -373 (-838$A_nT_n$)] in a large cohort of node-positive patients enrolled on a multicenter, cooperative group trial of adjuvant chemotherapy for breast cancer to determine whether other single nucleotide polymorphisms (SNP), combinations of SNPs, or haplotype are associated with breast cancer outcome, particularly among women with ER+ disease.

## Materials and Methods

We performed a retrospective cohort study using genomic DNA derived from hematologic circulating or bone marrow-derived stem cells and clinical data from breast cancer patients enrolled on Intergroup Trial 0121 (E2190/SWOG9061/CALGB 9496), a multicenter trial of high-dose versus standard dose adjuvant chemotherapy. Patients were included in the current study if they were enrolled in Intergroup Trial 0121 and had archival peripheral blood or bone marrow stem cells available for genomic DNA extraction and subsequent genotyping. Results of Intergroup Trial 0121 trial

**Note:** Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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**Table 1.** Characteristics of study population: genotyped group versus overall study cohort

| Characteristics | Genotyped cohort ($n$ = 346), median (interquartile range) | Full Intergroup Trial 0121 cohort ($n$ = 540), median (interquartile range) | $P$ |
|---|---|---|---|
| Age | 45 (39-50) | 44 (38-50) | 0.35* |
| Axillary lymph node positivity | 14 (11-19) | 14 (11-18) | 0.13* |
| Tumor size, cm | 3.5 (2.1-5.0) | 3.5 (2.1-5.0) | 0.90 |
| Median follow-up, y | 9.8 (8.3-11.2) | 9.7 (8.1-11.4) | 0.57* |
| | % | % | |
| Postmenopausal | 31 | 29 | 0.22* |
| Race, Caucasian | 90 | 89 | 0.58* |
| ER+ | 59 | 60 | 0.70* |
| PR+ | 56 | 59 | 0.12* |
| Lumpectomy | 17 | 19 | 0.10* |
| High-dose chemo treatment arm | 57 | 50 | <0.001 |
| 10 y DFS (%) | 39 | 43 | 0.02† |
| 10 y OS (%) | 45 | 48 | 0.09† |

*Pearson's $\chi^2$ test.
†Log-rank test.

have been published previously (14). Briefly, 540 patients with ≥10 positive lymph nodes received conventional adjuvant therapy with four cycles of cyclophosphamide (100 mg/m$^2$, orally, days 1-14), doxorubicin (30 mg/m$^2$, intravenously, days 1 and 8), and fluorouracil (500 mg/m$^2$, intravenously, days 1 and 8) followed by randomization to either observation or receipt of high-dose chemotherapy [cyclophosphamide (6 g/m$^2$) and thiotepa (800 mg/m$^2$) over a 4-day period] followed by stem cell rescue. Adjuvant tamoxifen was recommended for patients with ER+ tumors.

The protocol specified stem cell collection at the completion of standard CAF. Specimens not used for autologous reinfusion were stored at −80°C at the Eastern Cooperative Oncology Group (ECOG) Pathology Core Facility. The original Intergroup Trial 0121 consent form included language specifying that residual biological specimens would be used for future breast cancer research. Approval for the current study was obtained from the University of Pennsylvania Institutional Review Board and the ECOG Executive Committee.

The ECOG Pathology Core Facility at Northwestern University extracted DNA from hematologic stem cells with the EZ1 system (Qiagen). Genotyping was done at the University of Pennsylvania. DNA samples were identified only by their assigned pathology identification number and clinical information was assigned a case identification number. Laboratory personnel did not have access to clinical outcome data. Genotypes for the -174G>C, -597G>A (15), and -572G>C SNPs were determined by PyroSequencing (Biotage; ref. 16). The -597G>A and -572G>C assays were multiplexed; genotyping for -174G>C was a simplex assay. Genotyping for the -373A$_n$T$_n$ tract was done as described by Kelberman and colleagues (17) with modifications to PCR conditions.

The ECOG Statistical Center performed additional follow-up and delinked patient identifiers from the clinical data used in this analysis. Polymorphisms were analyzed for Hardy-Weinberg equilibrium (HWE) by Pearson's $\chi^2$ and Haldane's exact test. We formally tested for linkage disequilibrium by assessing for each pair of SNPs or haplotypes, $X$ and $Y$, with alleles $\{a, A\}$ for $X$ and $\{b, B\}$ for $Y$ (e.g., if $X$ = IL-6-174, then $\{a, A\} = \{C, G\}$), a standard measure of linkage disequilibrium $|D'| = |P(X = a, Y = b) - P(X = a)P(Y = b)| / M$, where $M = \min\{P(X = a)P(Y = B), P(X = A)P(Y = b)\}$ if $P(X = a, Y = b) > P(X = a)P(Y = b)$ and $M = \min\{P(X = a)P(Y = b), P(X = A)P(Y = B)\}$ if $P(X = a, Y = b) < P(X = a)P(Y = b)$. Because IL-6-373 has four levels, four different values of $|D'|$ were computed by treating each of the four levels in turn as the value $b$ (and combining the others in $B$). Fisher's exact test was used to determine if polymorphisms were in linkage equilibrium with one another and to adjust for baseline demographics and disease characteristics.

The primary endpoint was DFS, defined as time from randomization to earliest recurrence, new breast cancer, or death. The secondary endpoint was OS, defined as time from randomization to death (14). All survival times were censored at time of last contact or on August 1, 2005 if subjects were alive and disease-free at that time. Log-rank test $P$ values were used to determine associations between polymorphisms and DFS and OS. All associations of significance are based on two-sided tests. Cox proportional hazards regression models were also used for DFS and OS analysis. In the multivariable Cox models, we adjusted for metabolic genotypes, CYP3A4*1B and GSTM1, due to our previous finding that these cyclophosphamide-metabolizing genotypes were associated with outcome in this data set.[9]

Analyses were done to model the associations between IL-6 promoter haplotype and outcome using the HAPSTAT program (version 3.0).[10] We used the methods of Lin and Zeng (18–20), which employs the Cox proportional hazards model (21) to generate maximum likelihood estimators and their variances in an unbiased, normally distributed, and statistically efficient manner. It uses a weighted mixture based on the haplotype frequencies. We first inferred individual haplotypes for each subject, considering only those with frequencies >1% in the modeling process. The frequency of each haplotype by race was estimated to determine if significant racial differences were present. To understand whether potential haplotype effects differed by race, an approach using the combined data set but assuming HWE only within each ethnic group was done, as HAPSTAT does not support estimating haplotype distributions separately within subsets of the data. Due to the low level of phase ambiguity, we expect that there is no bias involved. Finally, we analyzed just the White race subset alone, because the other racial groups are too small to allow separate estimation of effects on DFS. $P$ values and estimated effects from these models were calculated and the tests and estimates for the effect of each haplotype are reported separately (that is, without adjustment for the effects of the other haplotypes).

[9] P.P. Gor, R.J. Gray, P. Gimotty, and colleagues. Drug metabolizing enzyme polymorphisms and survival outcomes in node positive breast cancer patients receiving adjuvant chemotherapy on Eastern Cooperative Oncology Group Protocol 2190/Intergroup 0121, submitted for publication.
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## Results

A total of 433 DNA samples from E2190 archival bone marrow or peripheral blood stem cell specimens were obtained from the ECOG Pathology Coordinating Office. Of these, 52 samples did not have matches to ECOG clinical case numbers, and 31 were duplicate samples. For 4 of these 31 pairs of samples, at least one of the IL-6 genotypes was discordant and so was excluded from analysis. The discordant polymorphisms varied between these four cases, suggesting that this was not a failure of one of the genotype assays. The remaining 27 subjects with duplicate samples had concordant results and were included in this analysis. Thus, a total of 346 of 540 (64%) subjects enrolled on E2190 were included in this analysis.

The clinical and tumor characteristics of the current study subjects were compared with the overall study cohort (Table 1). Patients in the current study were more likely to be on the BMT arm of the study (57% versus 50%; $P < 0.001$) and had slightly shorter 10-year DFS (39 versus 43 months; $P = 0.02$) but did not otherwise differ significantly from those not genotyped with respect to race, age, menopausal status, ER status, involved lymph node number, or tumor size. This cohort was primarily Caucasian (90%), 30% were age <40 years, and more than two-thirds were premenopausal at the time of diagnosis.

Frequencies for the IL-6 promoter genotype and haplotypes were calculated, both overall and by race (Table 2). Genotype failure rates were 5% for 174G>C and 373AT repeat and 1% for 572G>C and 597G>A. The genotypic distributions and minor allele frequencies in our study were similar to those seen in published studies of other breast cancer populations (22, 23). Genotype frequencies are consistent with HWE within racial groups. There is strong evidence that the three SNPs are not in linkage equilibrium ($P < 0.0001$ for each pair, Fisher's exact test). Supplementary Table S1 shows estimated values of $|D'|$ based the joint distribution of the SNPs for Whites. For the haplotype distributions for Blacks and other races, $|D'| = 1$ for all pairs. Comparison of the distributions of the three SNPs with the IL-6-373 variable repeat shows that these similarly are not in linkage equilibrium. However, the 8A12T sequence appears to be strongly associated with the presence of the C allele for IL-6-173 and the A allele for IL-6-597. The presence of the 10A10T sequence appears to be associated with the presence of the C allele for IL-6-572. Associations between each variant and clinical characteristics showed that all four variants were significantly associated with race ($P < 0.001$ for each, Fisher's exact test). Only IL-6-597 genotype was associated with ER status (ER positivity rates 48% for AA, 57% for AG, and 66% for GG, respectively; $P = 0.04$). Both IL-6-597 and IL-6-373 genotypes were significantly associated with number of positive lymph nodes ($P = 0.03$ for each).

Thirteen distinct naturally occurring haplotypes were generated by HAPSTAT; five haplotypes occurred in >1% of the study population ($n = 331$), as shown in Table 2. Haplotypes are described in order (-597, -572, -373, -174) moving from 5′ to 3′ along the promoter sequence. The different genotypes show strong linkage (tests for linkage disequilibrium were highly significant for each pair). There is little difference by ER status but a substantial difference by race; therefore, we controlled for race in subsequent modeling of the effect of these haplotypes on DFS.

The 10-year DFS and OS for each genotype overall and by tumor ER status are shown in Table 3. In the group overall, only IL-6-174 genotype was associated with DFS although of borderline significance (GG versus CC/CG, 32.3 versus 43.7 months; $P = 0.06$) and not significantly associated with OS. However, among patients with ER+ tumors, IL-6-174, IL-6-597, and IL-6-373 SNPs were each significantly associated with DFS. Because these polymorphisms are very highly associated with each other, each has a roughly equivalent effect. This association carried over to OS only for

**Table 2.** IL-6 promoter genotype and haplotype frequencies

| Locus | Genotype | All ($n = 346$) | White ($n = 314$) | Black ($n = 25$) | Other ($n = 21$) |
|---|---|---|---|---|---|
| - 597 (7 failed) | AA | 61 (18) | 59 (19) | 0 (0) | 2 (14) |
| | AG | 145 (43) | 140 (46) | 2 (11) | 3 (21) |
| | GG | 133 (39) | 108 (35) | 16 (89) | 9 (64) |
| - 572 (5 failed) | CC | 5 (1) | 1 (<1) | 0 (0) | 4 (27) |
| | CG | 36 (11) | 30 (10) | 4 (21) | 2 (13) |
| | GG | 300 (88) | 276 (90) | 15 (79) | 9 (60) |
| - 373 (18 failed) | Any 8A12T | 199 (61) | 192 (64) | 2 (12) | 5 (38) |
| | No 8A12T | 129 (39) | 106 (36) | 14 (88) | 8 (62) |
| - 174 (18 failed) | CC | 57 (17) | 55 (19) | 0 (0) | 2 (14) |
| | CG | 146 (45) | 140 (47) | 3 (17) | 3 (23) |
| | GG | 125 (38) | 102 (34) | 15 (83) | 8 (62) |

| Estimated haplotype frequencies* (597-572-373[$A_nT_n$]-174) | All ($n = 344$) | White ($n = 310$) | Black ($n = 19$) | Other ($n = 15$) |
|---|---|---|---|---|
| G-C-[10/10]-G | 0.07 | 0.05 | 0.11 | 0.33 |
| A-G-[8/12]-C | 0.38 | 0.40 | 0.05 | 0.26 |
| G-G-[10/10]-G | 0.11 | 0.10 | 0.20 | 0.18 |
| G-G-[9/11]-G | 0.22 | 0.22 | 0.23 | 0.14 |
| G-G-[10/11]-G | 0.20 | 0.20 | 0.41 | 0.09 |

*Estimated frequency per HAPSTAT for the presence of the haplotype in the study population. Includes only those haplotypes that occurred in >1% of patients in the cohort for which genotype information is complete at all four loci.

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**Table 3.** Unadjusted 10-y DFS and OS by host genotype and tumor ER status

| Locus | Genotype | Overall | *P* | ER+ | *P* | ER- | *P* |
|---|---|---|---|---|---|---|---|
| 10 y DFS (SE) | | | | | | | |
| | | (*n* = 339) | | | | | |
| IL-597 | AA, AG | 42.4 (3.5) | 0.27 | 48.9 (5.0) | 0.007 | 34.5 (5.0) | 0.27 |
| | GG | 34.9 (4.2) | | 30.1 (5.0) | | 44.2 (7.4) | |
| | | (*n* = 341) | | | | | |
| IL-6-572 | CC, CG | 45.8 (7.9) | 0.72 | 53.1 (9.5) | 0.37 | 30.8 (12.8) | 0.37 |
| | GG | 38.3 (2.9) | | 38.3 (3.8) | | 38.1 (4.3) | |
| | | (*n* = 326) | | | | | |
| IL-6-373 | Any 8A12T | 42.1 (3.6) | 0.21 | 48.3 (5.1) | 0.01 | 34.2 (5.0) | 0.45 |
| | No 8A12T | 34.4 (4.2) | | 30.0 (5.1) | | 42.4 (7.2) | |
| | | (*n* = 326) | | | | | |
| IL-6-174 | CC, CG | 43.7 (3.6) | 0.06 | 49.3 (4.9) | 0.003 | 35.9 (5.2) | |
| | GG | 32.3 (4.2) | | 28.5 (5.2) | | 38.9 (7.2) | 0.76 |
| 10 y OS (SE) | | | | | | | |
| | | (*n* = 339) | | (*n* = 200) | | (*n* = 139) | |
| IL-597 | AA, AG | 48.8 (3.6) | 0.96 | 54.9 (4.9) | 0.09 | 41.4 (5.2) | 0.17 |
| | GG | 39.9 (4.7) | | 33.4 (5.8) | | 51.5 (7.7) | |
| | | (*n* = 341) | | (*n* = 201) | | (*n* = 140) | |
| IL-6-572 | CC, CG | 47.8 (8.6) | 0.66 | 54.8 (11.1) | 0.17 | 30.8 (12.8) | 0.09 |
| | GG | 44.5 (3.0) | | 43.6 (4.1) | | 45.6 (4.6) | |
| | | (*n* = 326) | | (*n* = 191) | | (*n* = 137) | |
| IL-6-373 | Any 8A12T | 48.7 (3.6) | 0.65 | 54.3 (5.0) | 0.10 | 41.3 (5.2) | 0.38 |
| | No 8A12T | 38.7 (4.7) | | 33.8 (5.9) | | 46.8 (7.6) | |
| | | (*n* = 326) | | (*n* = 194) | | (*n* = 134) | |
| IL-6-174 | CC, CG | 49.7 (3.6) | 0.49 | 55.2 (4.8) | 0.06 | 42.1 (5.3) | 0.41 |
| | GG | 37.1 (4.7) | | 31.8 (6.0) | | 45.8 (7.6) | |

IL-6-174, with GG genotype associated with worse survival than CC/CG (31.8 versus 55.2 months), although this was of only borderline significance ($P = 0.06$). There were no significant associations between genotype and either DFS or OS among patients with ER- tumors.

Because patients in the parent study were randomized between standard, anthracycline-based chemotherapy alone or with the addition of high-dose cyclophosphamide and thiotepa, effects within treatment groups were also examined. The only case in which there was a substantial difference between the two treatment arms was for IL-6-572 in the ER- subset; however, the small sample size in this subanalysis precluded formal association testing. Because there did not appear to be a significant effect by treatment arm, arms were combined for the remainder of the analyses.

Results of multivariable Cox proportional hazards modeling for individual SNPs are shown in Table 4. Of the baseline characteristics examined (Table 1), only age and race were significantly associated with outcome and were thus included in the adjusted analysis. Although there was a significant association of genotype with number of nodes, neither number of nodes nor tumor size is significantly associated with DFS or OS in this cohort. Further adjustment for number of nodes and tumor size had little effect on the odds ratios for either DFS or OS (data not shown). Furthermore, we adjusted for drug-metabolizing enzyme SNPs in CYP3A4*1B and GSTM1 as prespecified in the analysis. To assess for proportionality of hazards, we applied the Grambsch-Therneau test to the fully adjusted DFS model and found that ER showed significant evidence of nonproportionality ($P = 0.003$). Thus, a stratified multivariable Cox model was computed; the overall model was stratified on ER and separate models were fit to estimate the effects within the ER+ and ER- subsets. DFS is significantly worse for patients with ER+ tumors who have either -597 GG or -174 GG genotype as well as those with the -373[8A12T] variant compared with the reference groups. The -174 GG genotype was borderline significantly associated with worse OS as was the -373[8A12T] variant. Among patients with ER- tumors, none of the individual genotypes were significantly associated with DFS or OS. Tests of interaction between ER status and genotype in the models stratified on ER that included the main effect of genotype plus the interaction term showed significant interactions for each individual variant and ER status (ER × -597: Wald $P = 0.01$ for DFS and $P = 0.04$ for OS, ER × -572: $P = 0.21$ for DFS and $P = 0.03$ for OS, ER × -373: $P = 0.03$ for DFS and $P = 0.09$ for OS, and ER × -174: $P = 0.04$ for DFS and $P = 0.07$ for OS).

We also examined the effects of genotype on outcome in the ER+ subset when other genotypes were included as covariates in the model. First, the IL-6 -174, -597, and -373 genotypes are so strongly linked that it is not possible to separate their effects. That is, -174 GG versus other and -597 GG versus other disagree for only six ER+ cases with data on both, -174 GG versus other and -373 no 8A12T versus any 8A12T disagree for only six ER+ cases with both evaluated, and 597 GG versus other and -373 no 8A12T versus any 8A12T disagree for only two ER+ cases with both evaluated. In joint models, the information about the relative effects comes from these discordant cases, and there are too few of these cases for the estimates to be meaningful. Also, the high colinearity between the indicator variables for these factors means that variances of the estimates are large, and in the joint models for pairs of these variables, neither is individually significant, although joint effect of both is highly significant.

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**Table 4.** Adjusted DFS and OS by host genotype and tumor ER status

| Genotype | DFS | | OS | |
|---|---|---|---|---|
| | HR (95% CI) | *P* | HR (95% CI) | *P* |
| Overall | | | | |
| -597 GG/(AG or AA) | 1.12 (0.84-1.51) | 0.43 | 0.99 (0.73-1.36) | 0.97 |
| -572 GG/(GC or CC) | 1.19 (0.75-1.87) | 0.46 | 1.15 (0.71-1.86) | 0.57 |
| -373 8A12T/(no 8A12T) | 0.85 (0.64-1.15) | 0.29 | 0.95 (0.69-1.31) | 0.76 |
| -174 GG/(CG or CC) | 1.26 (0.94-1.70) | 0.12 | 1.09 (0.80-1.50) | 0.57 |
| ER+ | | | | |
| -597 GG/(AG or AA) | 1.60 (1.09-2.35) | 0.02 | 1.39 (0.93-2.10) | 0.11 |
| -572 GG/(GC or CC) | 1.63 (0.90-2.94) | 0.11 | 1.83 (0.95-3.50) | 0.07 |
| -373 8A12T/(no 8A12T) | 0.62 (0.42-0.92) | 0.02 | 0.69 (0.46-1.06) | 0.09 |
| -174 GG/(CG or CC) | 1.71 (1.16-2.52) | 0.007 | 1.48 (0.98-2.24) | 0.06 |
| ER- | | | | |
| -597 GG/(AG or AA) | 0.68 (0.41-1.14) | 0.15 | 0.62 (0.35-1.09) | 0.10 |
| -572 GG/(GC or CC) | 0.78 (0.38-1.60) | 0.49 | 0.54 (0.26-1.11) | 0.10 |
| -373 8A12T/(no 8A12T) | 1.26 (0.77-2.06) | 0.35 | 1.34 (0.79-2.27) | 0.28 |
| -174 GG/(CG or CC) | 0.86 (0.52-1.43) | 0.57 | 0.75 (0.44-1.29) | 0.30 |

NOTE: Adjusted for age (<40 versus 40-60 y), race (White versus Black versus other), CYP3A4 (any G allele versus A/A), and GSTM1 (null versus present).

Joint models fit for the effects of any of these three SNPs and -572 found that both are significant. For example, in the model with -174 and -572, the estimated hazard ratio [HR; 95% confidence interval (95% CI)] is 2.04 (1.37-3.05) for -174 ($P = 0.0005$) and 2.38 (1.23-4.60) for -572 ($P = 0.01$). This is essentially what drives the definition of the "good-risk" group in that it is very similar to the group with at least one C allele for -174 (or nearly equivalently, at least one A allele for -597) or at least one C allele for -572. The -373 genotype is needed to differentiate the small poor-risk group, but it seems appropriate to conclude that only one of -174 and -597 is needed.

Associations between haplotype (for those haplotypes with frequency >1%) and DFS were examined in the population with ER+ tumors (Table 5). First, the effect of each haplotype on DFS was examined separately, assuming an additive effect. Race (White versus Black versus other) was included in the models because there were differences in the distribution of the haplotypes for different racial groups, although it has little effect. Relative to other haplotypes, the A-G-[8/12]-C appears to be significantly protective (HR, 0.69; 95% CI, 0.52-0.91), whereas G-G-[10/11]-G is associated with significantly worse DFS (HR, 1.46; 95% CI, 1.02-2.09). The results assuming HWE and Hardy-Weinberg disequilibrium are nearly identical (the *P* values for the test of HWE in the general model are >0.50 for all models). The results for the analyses of the White race subset are also similar for the significant effects.

For the combined race group, assuming HWE, the additive model with all four haplotypes had a significant overall likelihood ratio test ($P = 0.006$ on 4 *df*). For Whites only, the additive model with all four haplotypes also had a significant overall likelihood ratio test ($P = 0.02$ on 4 *df*). The joint codominant model was also examined for the combined race group (the codominant model with all four haplotypes could not be fit for the White race subset).

**Table 5.** Associations between haplotype and DFS in ER+ group ($n = 203$)

| Haplotype* | HWE | | Hardy-Weinberg disequilibrium | | HWE Caucasian only | |
|---|---|---|---|---|---|---|
| | HR† (95% CI) | *P* | HR† (95% CI) | *P* | HR† (95% CI) | *P* |
| G-C-[10/10]-G | 0.68 (0.42-1.08) | 0.10 | 0.68 (0.42-1.08) | 0.10 | 0.92 (0.52-1.63) | 0.79 |
| A-G-[8/12]-C | 0.69 (0.52-0.91) | 0.009 | 0.69 (0.52-0.91) | 0.009 | 0.64 (0.48-0.87) | 0.004 |
| G-G-[10/10]-G | 1.24 (0.81-1.91) | 0.32 | 1.24 (0.81-1.91) | 0.32 | 1.36 (0.85-2.19) | 0.20 |
| G-G-[9/11]-G | 1.31 (0.94-1.84) | 0.11 | 1.31 (0.93-1.84) | 0.12 | 1.22 (0.86-1.73) | 0.27 |
| G-G-[10/11]-G | 1.46 (1.02-2.09) | 0.04 | 1.46 (1.02-2.10) | 0.04 | 1.49 (1.03-2.16) | 0.03 |

NOTE: Includes only those with sufficient frequency for modeling. All other haplotypes distributed over 13 patients; thus, outcome assessment was done in 331 patients.

*Denoted 597-572-373[$A_n/T_n$]-174.

†Estimate is for effect of each copy.

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Comparing the model with codominant effects for the four haplotypes (plus race) with the model with only race, there is an overall significant haplotype effect ($P < 0.0001$, likelihood ratio test on 8 *df*). Dropping haplotypes from this model in a backwards elimination scheme, the first haplotype eliminated is G-G-[10A10T]-G, which had $P = 0.58$ in the joint model. The least significant haplotype of the three remaining is G-C-[10A10T]-G, for which the 2 *df* likelihood ratio test has $P = 0.07$. However, the effect is very consistent with additivity, and the 1 *df* test based on an additive model is significant. This model suggests combining the genotypes into three groups based on the following rationale. First, both A-G-[8/12]-C and G-C-[10/10]-G are associated with better DFS relative to the reference set. We therefore created a single "good-risk" group consisting of subjects estimated to have at least one copy of one of these two haplotypes. The set of genotypes associated with at least one copy of these haplotypes (designated "group A") includes (AA, GG, 8A12T/8A12T, CC), (AG, GG, 8A12T/9A11T, CC), (AG, CG, 8A12T/9A11T, CG), (AG, GG, 8A12T/10A11T, CG), (AG, GG, 8A12T/10A10T, CG), (AG, GG, 8A12T/9A11T, CG), (GG, CC, 10A10T/10A10T, GG), (GG, CG, 10A10T/10A11T, GG), (GG, CG, 10A10T/10A10T, GG), and (GG, CG, 10A10T/9A11T, GG). Another group, considered "poor-risk" (designated "group C"), consists of the six cases with two copies the G-G-[10A/11T]-G haplotype and has the worst DFS of the group, with early recurrences from 0.5 to 3.5 years from study entry. A third group (designated "group B") consists of the subjects whose genotypes are not in the other two groups, which for the set of 199 cases analyzed above consists of genotypes (GG, GG, *, GG) and (GG, CG, *, GG), where * can be any combination of 10A10T, 10A11T, and 9A11T (except the combination from the group C genotype).

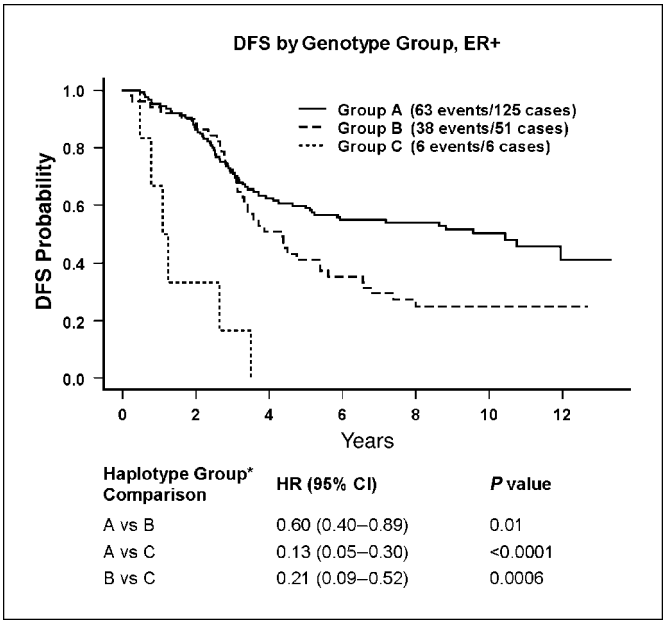
Kaplan-Meier estimates of DFS for these three groups are shown in Fig. 1. The plot shows a dose-response effect of haplotype group. Patients in group A with the "good-risk" haplotype have the best outcomes, those in group C with the "poor-risk" haplotype have the worst outcomes, and those in group B have intermediate outcomes. The pair-wise comparisons (noted below the figure) for all groups are significant, suggesting that IL-6 promoter haplotype identifies distinct prognostic groups within the ER+ subset. Notably, these prognostic groups are significantly associated with race, with a disproportionately greater percentage of Blacks making up the poor-risk group compared with the other two groups (33% group C versus 12.5% group B versus 3% group A; $P_{exact} = 0.03$).

## Discussion

We have shown that several different IL-6 promoter genotypes and a specific haplotype have significantly worse outcomes than their counterparts. This study confirms and extends our earlier work (13) in a much larger and multicenter clinical cohort. DFS was significantly worse for those with the -597 GG or -174 GG genotype or the G-G-[10A/11T]-G haplotype. For the latter, Kaplan-Meier plots show that all patients in this group relapsed by 24 months from diagnosis. This poor-risk haplotype, G-G-[10A/11T]-G, was quite common overall (estimated frequency, 0.20) and twice as frequent among Blacks (estimated frequency, 0.41).

These data shed new light on previous studies in which serum levels of IL-6 have been shown to be elevated in cancer patients relative to noncancer controls (24–26). In a more recent study comparing IL-6 levels in normal, early breast cancer, and metastatic breast cancer patients, Benoy and colleagues (27) found that serum IL-6 concentration was significantly higher in patients with breast cancer compared with healthy controls ($P < 0.0001$); median IL-6 serum levels were ~10 times higher in patients with metastatic breast cancer compared with those with locoregional disease (6.0 versus 0.7 pg/mL, respectively). Additional studies in metastatic breast cancer patients have shown high IL-6 serum levels to be associated with aggressive disease and poor clinical outcome. Zhang and colleagues showed that serum IL-6 levels are higher in patients with more numerous metastatic sites and poorer survival (28). Several other investigators have found associations between high serum IL-6 levels and poor response to breast cancer therapy (29), including resistance to both chemotherapy (30) and hormonal therapy (31).

However, because there is tremendous intraindividual variation in IL-6 spot levels, even in nonpathologic circumstances, and other factors, including medications, age, and nutritional status, may affect IL-6 level, we sought to determine if genotype would be a more stable and robust marker for outcome. Data to date clearly support the functional significance of the -174G>C polymorphism in controlling gene transcription and subsequent serum levels of IL-6 and several studies have linked IL-6-174G>C genotype to serum levels both in normal subjects and in those suffering either chronic disease or acute illness (32–34). Moreover, work by Terry and colleagues (12) suggests that there are multiple functional SNPs in the promoter region and that these polymorphisms have additive effects on gene expression. Transfection studies in HeLa cells and the ECV304 cell line showed that more than one of the polymorphisms was functional, the polymorphisms do not act independently, and one polymorphism influences the functional effect at the other polymorphism's site. Haplotypes in the ECV304 cell line exhibited functional differences and transcription was increased in the -597G, -572G, -373 9A11T, -174G haplotype and




| Haplotype Group* Comparison | HR (95% CI) | *P* value |
|---|---|---|
| A vs B | 0.60 (0.40–0.89) | 0.01 |
| A vs C | 0.13 (0.05–0.30) | <0.0001 |
| B vs C | 0.21 (0.09–0.52) | 0.0006 |

**Figure 1.** *Group A*, subjects with at least one copy of either (G-C-[10A/10T]-G) or (A-G-[8A/12T]-C). *Group B*, subjects whose genotypes are not in the other two groups, which for the set of 199 cases analyzed above consists of genotypes of (GG, GG, [*], GG) and (GG, GG, *, CG), where * can be any combination of (10A/10T), (10A/11T), and (9A/11T) (except the combination from the group C genotype). *Group C*, patients with two copies of (G-G-[10A/11T]-G).

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decreased transcription in the -597A, -572G, -373 8A12T, -174G. Our study findings are consistent with these preclinical and clinical observations, showing that high-production SNPs are associated with worse outcomes in breast cancer patients at high risk of relapse and that haplotype is a robust predictor of outcome.

Our finding that these effects are limited to those patients with ER+ tumors provides further support for the hypothesis that IL-6 exerts its effect on breast cancer cells at least in part through hormonal pathways. Cytokines, such as IL-6 and tumor necrosis factor-α, have an important role in regulating estrogen synthesis in peripheral tissues, including normal and malignant breast tissues (35). *In vitro*, the activities of aromatase, estradiol, 17β-hydroxysteroid dehydrogenase, and estrone sulfatase are all increased by IL-6 and tumor necrosis factor-α. We hypothesize that patients with ER+ tumors and poor-risk haplotypes are likely to fail despite hormonal therapy because of strong constitutive stimulation of aromatase, overwhelming the blockade by aromatase inhibitors in postmenopausal women or the blockade of ER receptors in those receiving tamoxifen, through competitive inhibition. We were unable to formally test this hypothesis in the current trial, as tamoxifen use was not documented in the trial participants despite being dictated by the protocol. However, additional studies are ongoing to address this issue and clarify whether IL-6 SNPs are prognostic or simply predictive of resistance to endocrine therapy. Finally, our observation that the "poor-risk" haplotype was significantly more prevalent in Black patients bears further study in a larger Black population. Although the numbers in the current study are small, these findings, if confirmed, may shed light on an additional mechanism by which Black patients with breast cancer suffer disproportionately poor outcomes compared with their White counterparts (36).

Several limitations must be considered in interpreting the current study. Although tamoxifen was "recommended" for all subjects with ER+ tumors in the parent clinical trial, no data were collected on patients in this trial with regard to prescription for tamoxifen or adherence among those prescribed the medication. However, it would be expected from results of other trials and population-based studies that this is likely to be <100% (37, 38), although the magnitude of this reduction is impossible to estimate in this study cohort of individuals at extremely high risk of recurrence. Based on our hypothesis that increased IL-6 transcription increases aromatase activity, one could postulate that this effect might be enhanced by the absence of tamoxifen. However, this is likely to reflect the "real world," as tamoxifen nonadherence continues to be an issue. Whether IL-6 contributes to the experience of adverse drug effects, such as hot flashes, further contributing to nonadherence, is unknown.

The grouping of haplotypes by outcome is clearly exploratory, and statistical differences in these groups should be viewed as such. However, understanding differences in outcome by haplotype is critically important in identifying subpopulations for whom current therapy is insufficient and thus provides important information in selecting appropriate patients for testing of new agents directed at IL-6-related pathways.

Nonetheless, this is the largest study to date to provide strong evidence of a role for host IL-6 genotype in modulating outcomes in ER+ breast cancer. Further studies are necessary to determine whether these differences are due to immune, hormonal, or cell signaling effects in ER+ breast cancer cells, potentially leading to the development of IL-6-targeted approaches to therapy and the ability to identify patients in whom these treatments are likely to be necessary and effective.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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# Host Genetic Variants in the *Interleukin-6* Promoter Predict Poor Outcome in Patients with Estrogen Receptor-Positive, Node-Positive Breast Cancer

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